



UNIVERSITI PUTRA MALAYSIA

**EXPRESSION OF TUMOUR-ASSOCIATED ANTIGENS AND
CHARACTERISTICS OF T CELL RESPONSES IN BREAST
CARCINOMA**

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**EXPRESSION OF TUMOUR-ASSOCIATED ANTIGENS AND
CHARACTERISTICS OF T CELL RESPONSES IN BREAST CARCINOMA**

LEONG POOI POOI

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia
in Fulfilment of Requirements for the Degree of Master of Science**

March 2005



Specially dedicated to,

My mother, husband, sister and brother

For their love, understanding, encouragement and patience

Good luck to you all.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment
of the requirements for degree of Master of Science

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EXPRESSION OF TUMOUR-ASSOCIATED ANTIGENS AND CHARACTERISTIC OF T CELL RESPONSES IN BREAST CARCINOMA

By

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March 2005

Chairman : Professor Seow Heng Fong, PhD

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Breast cancer is the most common cancer among women in Malaysia. The standard conventional clinical management procedures use chemotherapy, radiotherapy and mastectomy. In the past decade, intense research towards the use of T-cell based immunotherapy as a treatment alternative has been made. The goals of our study are first to identify some of the tumour-associated antigens present in our tumour specimens from patients with infiltrating ductal carcinoma (IDC) of the breast, followed by antigenic peptide selection in order to develop an *in vitro* T-cell based cytotoxicity assay. At the same time, we also identified immunophenotypes of the tumour infiltrating lymphocytes (TILs) in the breast tumours. Isolated peripheral blood mononuclear cells (PBMCs) from patients with IDC were specifically stimulated with three combinations of cytokines and antibodies that were specific to the co-stimulatory molecule and HLA-A02 restricted antigen-specific peptides. Stimulated PBMCs were then used as effector cells in cytotoxicity assay using calcein-AM in which the MCF-7 breast adenocarcinoma cell line served as the target cells. Phenotypic investigation of tumour cell suspension was carried out by using specific lymphocyte cell differentiation markers. By using paraffin-embedded breast

tissues (n=49), immunohistochemistry studies showed significant expression of survivin (80.1%, $p<0.001$), cytoplasmic MUC-1 (38.3%, $p<0.05$) and membranous MUC-1 (63.8%, $p<0.001$) in the tumour area as compared to the apparently normal adjacent tissues. These results provided a guide for antigenic peptide selection for stimulating the T cells from the blood of the patients. Together in the presence of rIL-2 and rIL-7, 4 out of 9 peripheral blood mononuclear cells (PBMCs) from the patients responded to either survivin-derived peptide (S2) or Her2/neu specific peptide (H2) in a HLA-A02 restricted manner in order to produce sufficient amounts of effector cells for the subsequent cytotoxic assay. As effector/target (E/T) ratio increased, cytolytic activity of the effector cells became more efficient. For immunophenotypic analysis, CD8⁺ TILs at $23.4 \pm 2.1\%$ was found to be the major population in TILs and the presence of its effector counterpart, CD8⁺CD28⁺ TILs significantly correlated with low incidence of metastasis ($p<0.05$). At the same time, we noticed the predominance of CD4⁺CD25⁺ regulatory T cells (Treg) at $55.9 \pm 3.9\%$ in the Treg pool and its presence was significantly found in post-menopausal patients ($p<0.05$). In conclusion, survivin and MUC-1 (cytoplasmic and membranous) were over-expressed in breast cancer tissues. Further investigations are needed to determine the reasons as to why only a portion of PBMCs from the patients (4/9) responded to the specific peptide-based stimulation and showed effective cytolytic activity towards the target breast adenocarcinoma MCF-7 cell line. It is possible that other cytokine cocktails are needed to enhance the cytolytic property of the PBMCs. We also found that infiltration of effector TILs, CD8⁺CD28⁺, significantly reduced the metastatic event. Lastly, we noted that older women (≥ 50 years old) tend to possess higher amount of CD4⁺CD25⁺ Treg in TILs as compared to the younger patients (< 50 years old). The higher CD4⁺CD5⁺ Treg

in TILs may implicate poor disease outcome in older patients. We proposed that these Treg cells contribute to tumour escape mechanism.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Sarjana Sains

TINDAK BALAS SEL T TERHADAP ANTIGEN YANG BERKAITAN DENGAN KANSER PAYU DARA

Oleh

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Barah payudara merupakan kanser yang paling umum di kalangan wanita di Malaysia. Kaedah-kaedah perubatan klinikal yang biasa digunakan adalah kimiaterapi, radioterapi dan pembedahan. Dalam dekad yang lalu, banyak penyelidikan terhadap penggunaan imunoterapi sebagai kaedah perubatan alternatif telah dijalankan. Tujuan kajian ini adalah, pertama, untuk mengenalpasti beberapa antigen yang berkaitan dengan kanser (TAA) dalam specimen-specimen pesakit yang mengalami penyebaran sel-sel kanser ke salur duktur payudara (IDC), dan kedua, untuk memilih peptid antigen agar esei sitotoksik sel T dapat dilaksanakan. Pada masa yang sama, kami juga mengenalpasti imunofinotip dalam sel-sel limfosit yang tersebar dalam kanser payudara (TILs). Sel-sel mononuklear periperal darah (PBMC) yang diasingkan dari pesakit yang mengalami penyebaran sel-sel kanser ke salur duktur payudara telah dirangsangkan dengan menggunakan kombinasi sitokin dan antibodi yang spesifik terhadap perangsangan berpandu dan peptid spesifik terhadap antigen terhad HLA-A02. PBMC yang terangsang digunakan sebagai sel efektor dalam esei sitotoksik calcein-AM di mana sel kultur

adenokarsinoma payudara MCF-7 digunakan sebagai sel target. Penyelidikan finotip terhadap sel-sel limfosit yang tersebar dalam kanser payudara dijalankan dengan menggunakan sel-sel kanser yang telah diceraikan oleh enzim dan tanda-tanda perbezaan sel limfosit yang spesifik. Dalam tisu kanser payudara yang berparafin (n=49), kajian imunohistokimia memaparkan ekspresi yang nyata terhadap survivin (80%, $P<0.001$), MUC-1 di sitoplasma (38.3%, $P<0.05$) and MUC-1 di membran (63.8%, $P<0.001$) di dalam kawasan sel-sel kanser berbanding dengan sel-sel normal yang bersebelahan. Keputusan ini memberi panduan dalam pemilihan antigen peptid untuk merangsang sel-sel T limfosit daripada darah pesakit tersebut. Dengan kehadiran rIL-2 dan rIL-7, empat daripada sembilan pesakit mempunyai sel-sel mononuklear perifer darah bertindakbalas terhadap peptide survivin atau peptid spesifik Her2/neu dalam keadaan HLA-A02 dihadkan agar dapat menghasilkan sel-sel efektor yang cukup untuk esei sitotoksik yang seterusnya. Apabila ratio efektor/target (E/T) meningkat, sel efektor semakin cekap menjalankan aktiviti sitolisis. Dalam imunofenotip analisa, sebanyak 23.4 ± 2.1 % CD8+TILs merupakan kumpulan yang terbesar dalam TILs dan, dengan nyata sekali, kehadiran sel efektor CD8+CD28+TILs berkait rapat dengan insiden metastasis yang rendah ($P<0.05$). Pada masa yang sama, kami mendapati sel CD4+CD25+regulasi T (Treg) mendominasi kumpulan Treg dengan sebanyak 55.9 ± 3.9 % dan kehadirannya hanya nyata dalam pesakit lebih tua (≥ 50 tahun) ($p<0.05$). Sebagai kesimpulan, survivin dan MUC-1 (dalam sitoplasma dan pada membran) adalah terlebih ekspres dalam tisu kanser payudara. Penyelidikan yang lebih mendalam harus dilakukan untuk mengetahui sebab-sebab kenapa hanya sebahagian daripada pesakit (4/9) bertindakbalas terhadap rangsangan spesifik peptid dan menonjolkan aktiviti sitolisis yang berkesan terhadap sel kultur adenokarsinoma payudara MCF-7.

Campuran beberapa sitokin mungkin akan meningkatkan aktiviti sitolisis PBMCs terhadap sel kanser. Kami juga mendapati sel efektor CD8+CD28+TILs berkesan mengurangkan insiden metastasis. Akhir sekali, kami mendapati wanita yang lebih tua cenderung memiliki jumlah sel CD4+CD25+Treg yang tinggi dalam Treg jika dibandingkan dengan pesakit lebih muda usianya. Jumlah sel CD4+CD25+Treg yang banyak mengimplikasi prognosi yang lemah dalam kalangan wanita susut haid. Kami mencadangkan bahawa Treg tersebut menyumbang kepada mekanisme di mana sel kanser terbebas daripada sistem imun.

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I certify that an Examination Committee met on 30th March 2005 to conduct the final examination of Leong Pooi Pooi on her Master of Science thesis entitled "Expression of Tumour-Associated Antigens and Characteristics of T-Cell Responses in Breast Carcinoma" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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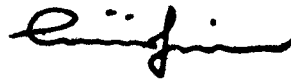
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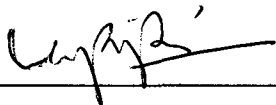
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DECLARATION

I hereby declare that the thesis is based on my original work except for equations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.



LEONG POOI POOI

Date: 9 August 2005

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LIST OF ABBREVIATIONS

| | |
|-----------------|---|
| α | alpha |
| β | beta |
| γ | gamma |
| δ | delta |
| % | percentage |
| °C | Degree of Celsius |
| μg | microgram |
| ACD | acid citrate dextrose |
| AICD | activated-induced cell death |
| AMC | atypical medullary carcinoma |
| APC | allophycocyanin |
| APCs | antigen presenting cells |
| APES | aminopropyltrimethoxysilane |
| BCG | bacilli Calmette-Guerrin |
| BCS | breast conservation surgery |
| bp | base pair |
| BRAC | breast cancer susceptibility protein |
| BSA | bovine serum albumin |
| Calcein-AM | calcein- acetoxymethyl |
| CD | cluster of differentiation |
| cm | centimeter |
| CO ₂ | carbon dioxide |
| COX-2 | cyclooxygenase-2 |
| CTLs | cytotoxic T lymphocytes |
| Cy-chrome | cyanine-chrome |
| DAB | diaminobenzidine tetrahydrochloride |
| DCIS | ductal carcinoma in situ |
| DCs | dendritic cells |
| DMSO | dimethylsulphoxide |
| DNA | deoxyribonucleic acid |
| DTH | delayed type hypersensitivity |
| EGFR | epithelial growth factor receptor |
| ELISA | enzyme-linked immunosorbent assay |
| ER | oestrogen receptor |
| E/T | effector/target ratio |
| EthD | ethidium bromide homodimer |
| FADD | Fas-associated protein with death domain |
| FASL | Fas ligand |
| FBS | fetal bovine serum |
| Fc | forward scatter |
| FITC | fluorescein isothiocyanate |
| FL | filter |
| FLICE | FADD homologous Interleukine-1 beta converting enzyme/ <i>Caenorhabditis elegans</i> cell-death protein 3- like protease |
| FOXP | Foxhead/ winged-helix |
| GITR | glucocorticoid induced tumour necrosis receptor |

| | |
|-------------------|--|
| GSK | glycogen synthase kinase |
| Her2/neu | human epidermal growth factor 2/ neu |
| HLA | human leukocyte antigen |
| HPV | human papillomavirus |
| HRT | hormone replacement therapy |
| HUKM | Hospital Universiti Kebangsaan Malaysia |
| IAP | inhibitory of apoptosis |
| ICAM-1 | intracellular cell adhesion molecules-1 |
| IDC | infiltrating ductal carcinoma |
| IFN- γ | interferon gamma |
| IL- | interleukin |
| iNKR _s | inhibitory NK receptor |
| LAK | lymphocyte activated killer |
| LMP | latent membrane protein |
| LOH | loss of heterozygosity |
| MAGE | melanoma-associated antigen |
| MAPK | mitogen-activated protein kinase |
| MART-1 | melanoma antigen recognized by T cell-1 |
| MC | medullary carcinoma |
| MECL-1 | multicatalytic endopeptidase complex like-1 |
| MHC | major histocompatibility complex |
| ml | milliliter |
| mm | millimeter |
| MUC | mucin |
| NCCN | National Comprehensive Cancer Network |
| NKT | natural killer T |
| NSABP P1 | National Surgical Adjuvant Breast and Bowel project –Phase 1 |
| PBMCs | peripheral blood mononuclear cells |
| PBS | phosphate buffered saline |
| PCR-SSP | polymerase chain reaction- sequence specific primer |
| PGE ₂ | prostaglandin E2 |
| PE | phycoerythrin |
| PerCp | peridinin chlorophyll protein |
| PI | propidium iodide |
| PI3K-Akt | phosphatidylinositol 3- kinase/ Akt |
| PR | progesterone receptor |
| rIL- | recombinant interleukin |
| RNAi | interference ribosomal nucleic acid |
| RPMI 1640 | Roswell Park Memorial Institute 1640 |
| TAA _s | tumour-associated antigens |
| TAE | Tris-acetate-EDTA |
| TAP | transporter associated with antigen processing |
| TCR | T cell receptor |
| TGF | transforming growth factor |
| Th | T helper |
| TILs | tumour infiltrating lymphocytes |
| TLR | toll-like receptor |
| TMC | typical medullary carcinoma |
| TNFR | tumour necrosis factor receptor |
| TRAIL | tumour necrosis factor receptor- related apoptosis inducing ligand |

| | |
|------|------------------------------------|
| Treg | regulatory T lymphocyte |
| U | international unit |
| VEGF | vascular endothelial growth factor |
| VNTR | variable number of tandem repeats |